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(54) Title: APTAMERS SPECIFIC FOR THROMBIN AND METHODS OF USE

(57) Abstract

Oligonucleotide sequences that mediate specific binding to thrombin and optionally contain modified bases, sugars, or sugar linkages are disclosed. Single-stranded DNA oligomers are obtained that bind thrombin and inhibit its function *in vitro* and *in vivo*. The thrombin binding oligomers are useful for therapeutic, diagnostic and manufacturing purposes. An improved method for identifying these oligomers is also described, involving complexation of the support-bound thrombin with a mixture of oligonucleotides containing random sequences under conditions wherein a complex is formed with the specifically binding sequences, but not with the other members of the oligonucleotide mixture. The thrombin-oligonucleotide complexes are then separated from both the support and the uncomplexed oligonucleotides and the complexed members of the oligonucleotide mixture are recovered from the separated complex and subsequently amplified using standard techniques.

APTAMERS SPECIFIC FOR THROMBIN AND METHODS OF USE

Technical Field

This invention is in the field of rational drug design using biomolecule targeting and aptamer development. The invention discloses and claims methods for making aptamers to thrombin and the aptamers resulting therefrom which may be applied broadly to diagnostics and therapeutics. More specifically, this invention is related to aptamers that bind to thrombin and interfere with its normal biological function, and therapeutic uses for these aptamers.

Background and Related Art

Specifically Binding Oligonucleotides. Conventional methods of detection and isolation of proteins and other molecules have employed antibodies and the like which specifically bind such substances. Recently, however, the *de novo* design of specifically binding oligonucleotides for non-oligonucleotide targets that generally bind nucleic acids has been described. See, e.g., Blackwell, T.K., et al., Science (1990) 250:1104-1110; Blackwell, T.K., et al., Science (1990) 250:1149-1152; Tuerk, C., and Gold, L., Science (1990) 249:505-510; Joyce, G.F., Gene (1989) 82:83-87. Such oligonucleotides have been termed "aptamers" herein. Tuerk and Gold describe the use of an *in vitro* selection and enrichment procedure. In this method, a pool of RNAs that are completely randomized at specific positions is subjected to selection for binding by a desired nucleic acid-binding protein which is then bound to a nitrocellulose filter. The bound RNAs then are recovered and amplified as double-stranded DNA that is competent

PCR and transcribed into RNA. It was thought that the complexity of the pool was reduced in the amplification/transcription steps to approximately 10^{13} different sequences. The pool was then applied to an affinity column containing the dye and the bound sequences subsequently eluted, treated with reverse transcriptase and amplified by PCR. The results showed that about one in 10^{10} random sequence RNA molecules folds in such a way as to bind specifically to the ligand.

Thiesen, H.-J., and Bach, C., Nucleic Acids Res. (1990) 18:3203-3208, describe what they call a target detection assay (TDA) to determine double-stranded DNA binding sites for putative DNA binding proteins. In their approach, a purified functionally active DNA binding protein and a pool of random double-stranded oligonucleotides which contain PCR primer sites at each end were incubated with the protein. The resulting DNA complexes with the protein (in their case, the SP-1 regulatory protein) were separated from the unbound oligomers in the random mixture by band-shift electrophoresis and the SP-1 bound oligonucleotides were rescued by PCR and cloned, and then sequenced.

None of the cited references describe the use of single-stranded DNA as an appropriate material for generating aptamers. The use of DNA aptamers has several advantages over RNA including increased nuclease stability (Shaw, J.P. et al., Nuc Acid Res (1991) 19:747-750), in particular plasma nuclease stability, and ease of amplificatio: by PCR or other methods. RNA generally is converted to DNA prior to amplification using reverse transcriptase, a process that is not equally efficient with all sequences, resulting in loss of some aptamers from a selected pool.

of prostacyclin, platelet-activating factor and other factors; (vi) induces neutrophil adherence to vessel walls; (vii) stimulates vascular endothelial cell adhesion phenotype; and (viii) generates activated protein C by cleavage of protein C.

Mitogenic activity of thrombin is exerted through binding to thrombin receptors (Coughlin, S.R., et al, J. Clin. Invest., (1992) 89:351-355). Platelet aggregation, which plays a major role in arterial thrombosis is largely dependent on the function of thrombin (Hanson, S.R., et al, Proc. Natl. Acad. Sci. USA, (1988) 85:3184-3188). Platelets carry functional thrombin receptors. Inflammatory responses can also be mediated by thrombin through stimulation of platelet activating factor (PAF) (Prescott, S., et al, Proc. Natl. Acad. Sci. USA, (1984) 81:3534-3538. PAF promotes adhesion of neutrophils to endothelial matrix, leading to degranulation of the neutrophils and an associated inflammatory response.

Disclosure of the Invention

The identification of oligonucleotides that specifically bind to thrombin, which does not normally bind to RNA or DNA, has now been demonstrated. The thrombin aptamers bind to thrombin and inhibit both its catalytic activity in converting fibrinogen to fibrin and its platelet aggregating activity. The aptamers are potent inhibitors of thrombin function and represent a new cla of pharmaceutical agents for modulation of the activity of this protease. The molecules of this invention may be utilized in compositions and methods for inhibiting any thrombin-mediated or thrombin-associated process or function. Pharmaceutical compositions containing these molecules, as well as methods of treatment or prophylaxis of vascular diseases,

having random sequences may also contains a consensus sequence known to bind to thrombin.

In yet another aspect, this invention is directed to single-stranded deoxyribonucleotides that bind specifically to thrombin. It has been heretofore thought that the three-dimensional structure of double-stranded DNA limited the structural diversity of the molecule. The inventors herein are unaware of any prior demonstration of structural diversity for single- or double-stranded DNA sufficient to provide the range of conformations necessary to provide aptamers to biomolecules. For example, known RNA structures, such as pseudoknots, have not been described for single-stranded DNA.

In other aspects, the invention is directed to oligonucleotides which contain sequences identified by the above methods, and to oligonucleotide sequences which bind specifically to thrombin. In still another aspect, the invention is directed to complexes comprising the thrombin target substance and specifically bound oligomer.

In still other aspects, the invention is directed to oligomers which contain sequences that bind specifically to thrombin target substances and inhibit its normal biological function, and to the use of these oligomers in therapy, diagnostics, and purification procedures.

In yet a further aspect, this invention is directed to oligomers which contain sequences that bind specifically to thrombin and inhibits its normal biological function, and which also contain one or more modified bases, sugars, or sugar linkages, and to the use of these oligomers in therapy, diagnostics, and purification procedures.

used in diagnosis by employing them in specific binding assays.

For application in such various uses, the aptamers of the invention may be coupled to auxiliary substances that enhance or complement the function of the aptamer. Such auxiliary substances include, for example, labels such as radioisotopes, fluorescent labels, enzyme labels and the like; specific binding reagents such as antibodies, additional aptamer sequence, cell surface receptor ligands, receptors per se and the like; toxins such as diphtheria toxin, tetanus toxin or ricin; drugs such as antiinflammatory, antibiotic, or metabolic regulator pharmaceuticals, solid supports such as chromatographic or electrophoretic supports, and the like. Suitable techniques for coupling of aptamers to desired auxiliary substances are generally known for a variety of such auxiliary substances, and the specific nature of the coupling procedure will depend on the nature of the auxiliary substance chosen. Coupling may be direct covalent coupling or may involve the use of synthetic linkers such as those marketed by Pierce Chemical Co., Rockford, IL.

As used herein, "specifically binding oligonucleotides" or "aptamers" refers to oligonucleotides having specific binding regions which are capable of forming complexes with thrombin in an environment wherein other substances in the same environment are not complexed to the oligonucleotide. The specificity of the binding is defined in terms of the comparative dissociation constants of the aptamer for thrombin as compared to the dissociation constant with respect to the aptamer and other materials in the environment or unrelated molecules in general. Typically, the Kd for the aptamer with respect to thrombin will be 2-fold, preferably 5-fold, more

assay conditions with the candidate material. Generally, for antibodies to crossreact in standard assays, the binding affinities of the antibodies for crossreactive materials as compared to thrombin should be in the range of 5-fold to 100-fold, generally about 10-fold.

Thus, aptamers which contain specific binding regions are specific with respect to unrelated materials and with respect to materials which do not normally bind such oligonucleotides such as nucleases and restriction enzymes. In general, a minimum of approximately 6 nucleotides, preferably 10, and more preferably 14 or 15 nucleotides, are necessary to effect specific binding. Aptamers of sequences as short as 6 bases have been shown to specifically bind and inhibit thrombin. The only apparent limitations on the binding specificity of the thrombin/oligonucleotide couples of the invention concern sufficient sequence to be distinctive in the binding oligonucleotide and sufficient binding capacity of thrombin to obtain the necessary interaction. Oligonucleotides of sequences shorter than 10, e.g., 6 mers, are feasible if the approp ate interaction can be obtained in the context of the environment in which the thrombin is placed. Thus, if there are few interferences by other materials, less specificity and less strength of binding may be required.

As used herein, "aptamer" refers in general to either an oligonucleotide of a single defined sequence or a mixture of said oligonucleotides, wherein the mixture retains the properties of binding specifically to thrombin. Thus, as used herein "aptamer" denotes both singular and plural sequences of oligonucleotides, as defined herein.

Structurally, the aptamers of the invention are specifically binding oligonucleotides, wherein "cligonucleotide" is as defined herein. As set forth

Alternative linking groups include, but are not limited to embodiments wherein a moiety of the formula P(O)S, ("thioate"), P(S)S ("dithioate"), P(O)NR'$_2$, P(O)R', P(O)OR^6, CO, or CONR'$_2$, wherein R' is H (or a salt) or alkyl (1-12C) and R^6 is alkyl (1-9C) is joined to adjacent nucleotides through -O- or -S-. Dithioate linkages are disclosed and claimed in commonly owned U.S. application no. 248,517. Substitute linkages that may be used in the oligomers disclosed herein also include nonphosphorous-based internucleotide linkages such as the 3'-thioformacetal (-S-CH$_2$-O-), formacetal (-O-CH$_2$-O-) and 3'-amine (-NH-CH$_2$-CH$_2$-) internucleotide linkages disclosed and claimed in commonly owned pending U.S. patent application serial nos. 690,786 and 763,130, both incorporated herein by reference. One or more substitute linkages may be utilized in the oligomers in order to further facilitate binding with complementary target nucleic acid sequences or to increase the stability of the oligomers toward nucleases, as well as to confer permeation ability. (Not all such linkages in the same oligomer need be identical.)

The term "nucleoside" or "nucleotide" is similarly generic to ribonucleosides or ribonucleotides, deoxyribonucleosides or deoxyribonucleotides, or to any other nucleoside which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. Thus, the stereochemistry of the sugar carbons may be other than that of D-ribose in one or more residues. Also included are analogs where the ribose or deoxyribose moiety is replaced by an alternate structure such as the 6-membered morpholino ring described in U.S. patent number 5,034,506 or where an acyclic structure serves as a scaffold that positions the base analogs described herein in a manner that permits efficient binding to target nucleic acid sequences or other

thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, queosine, 2-thiocytosine, 5-propyluracil, 5-propylcytosine, 5-ethyluracil, 5-ethylcytosine, 5-butyluracil, 5-butylcytosine, 5-pentyluracil, 5-pentylcytosine, and 2,6-diaminopurine.

In addition to the modified bases above, nucleotide residues which are abasic, i.e., devoid of a purine or a pyrimidine base may also be included in the aptamers of the invention and in the methods for their obtention.

The sugar residues in the oligonucleotides of the invention may also be other than conventional ribose and deoxyribose residues. In particular, substitution at the 2'-position of the furanose residue is particularly important.

Aptamer oligonucleotides may contain analogous forms of ribose or deoxyribose sugars that are generally known in the art. An exemplary, but not exhaustive list includes 2' substituted sugars such as 2'-O-methyl-, 2'-O-alkyl, 2'-O-allyl, 2'-S-alkyl, 2'-S-allyl, 2'-fluoro-, 2'-halo, or 2'-azido-ribose, carbocyclic sugar analogs, α-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside, ethyl riboside or propyl riboside.

Although the conventional sugars and bases will be used in applying the method of the invention, substitution of analogous forms of sugars, purines and pyrimidines can be advantageous in designing the final product. Additional techniques, such as methods of synthesis of 2'-modified sugars or carbocyclic sugar analogs, are described in Sproat, B.S. et al., Nuc Acid Res (1991) 19:733-738; Cotten, M. et al., Nuc Acid Res

described in, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202 and Saiki, R.K., et al., Science (1988) 239:487-491, and European patent applications 86302298.4, 86302299.2 and 87300203.4, as well as Methods in Enzymology (1987) 155:335-350. If RNA is initially used, the amplified DNA sequences are transcribed into RNA. The recovered DNA or RNA, in the original single-stranded or duplex form, is then used in another round of selection and amplification. After three to six rounds of selection/amplification, oligomers that bind with an affinity in the mM to μM range can be obtained and affinities below the μM range are possible. PCR may also be performed in the presence of thrombin.

Other methods of amplification may be employed including standard cloning, ligase chain reaction, etc. (See e.g., Chu, et al., U.S. Patent No. 4,957,858). For example, to practice this invention using cloning, once the aptamer has been identified, linkers may be attached to each side to facilitate cloning into standard vectors. Aptamers, either in single or double stranded form, may be cloned and recovered thereby providing an alternative amplification method.

Amplified sequences can be applied to sequencing gels after any round to determine the nature of the aptamers being selected by thrombin. The entire process then may be repeated using the recovered and amplified duplex if sufficient resolution is not obtained.

Amplified sequences can be cloned and individual oligonucleotides then sequenced. The entire process can then be repeated using the recovered and amplified oligomers as needed. Once an aptamer that binds specifically to thrombin has been selected, it may be recovered as DNA or RNA in single-stranded or duplex form using conventional techniques.

(1987) 6:287-291; Froehler, B., Tet Lett (1986) 27:5575-5578. Oligonucleotides may also be synthesized using solution phase methods such as triester synthesis, known in the art. The nature of the mixture is determined by the manner of the conduct of synthesis. Randomization can be achieved, if desired, by supplying mixtures of nucleotides for the positions at which randomization is desired. Any proportion of nucleotides and any desired number of such nucleotides can be supplied at any particular step. Thus, any degree of randomization may be employed. Some positions may be randomized by mixtures of only two or three bases rather than the conventional four. Randomized positions may alternate with those which have been specified. It may be helpful if some portions of the candidate randomized sequence are in fact known.

In one embodiment of the method of the invention, the starting mixture of oligonucleotides subjected to the invention method will have a binding affinity for thrombin characterized by a Kd of 1 μM or greater. Binding affinities of the original mixture for thrombin may range from about 100 μM to 10 μM to 1 μM but, of course, the smaller the value of the dissociation constant, the more initial affinity there is in the starting material for thrombin. This may or may not be advantageous as specificity may be sacrificed by starting the procedure with materials with high binding affinity.

By application of the method of the invention as described herein, improvements in the binding affinity over one or several iterations of the above steps of at least a factor of 50, preferably of a factor of 100, and more preferably of a factor of 200 may be achieved. As defined herein, a ratio of binding affinity reflects the ratio of Kds of the comparative complexes. Even more preferred in the conduct of the method of the invention

of the dissociation constant of the thrombin/aptamer complex.

Use of Modified Nucleotides and Oligonucleotides

In one embodiment of the invention method, the initial mixture of candidate oligonucleotides will include oligomers which contain at least one modified nucleotide residue or linking group.

If certain specific modifications are included in the amplification process as well, advantage can be taken of additional properties of any modified nucleotides, such as the presence of specific affinity agents in the purification of the desired materials.

In order for the modified oligomer to yield useful results, the modification must result in a residue which is "read" in a known way by the polymerizing enzyme used in the amplification procedure. It is not necessary that the modified residue be incorporated into the oligomers in the amplification process, as long it is possible to discern from the nucleotide incorporated at the corresponding position the nature of the modification contained in the candidate, and provided only one round of complexation/amplification is needed. However, many of the modified residues of the invention are also susceptible to enzymatic incorporation into oligonucleotides by the commonly used polymerase enzymes and the resulting oligomers will then directly read on the nature of the candidate actually contained in the initial complex. It should be noted that if more than one round of complexation is needed, the amplified sequence must include the modified residue, unless the entire pool is sequenced and resynthesized to include the modified residue.

Certain modifications can be made to the base residues in a oligonucleotide sequence without impairing

amplified mixture is used in a second round, this new mixture must contain the modification.

Of course, if the selected aptamer is sequenced and resynthesized, modified oligonucleotides and linking groups may arbitrarily be used in the synthesized form of the aptamer.

Inclusion of modified oligonucleotides in the methods and aptamers of the invention provides a tool for expansion of the repertoire of candidates to include large numbers of additional oligonucleotide sequences. Such expansion of the candidate pool may be especially important as the demonstration of binding to proteins, for example, in the prior art is limited to those proteins known to have the capability to bind DNA. Modifications of the oligonucleotide may be necessary to include all desired sequences among those for which specific binding can be achieved.

Thus, one preferred method comprises incubating thrombin with a mixture of oligonucleotides, wherein these oligonucleotides contain at least one modified nucleotide residue or linkage, under conditions wherein complexation occurs with some but not all members of the mixture; separating the complexed from uncomplexed oligonucleotides, recovering and amplifying the complexed oligonucleotides and optionally determining the sequence of the recovered nucleotides. In an additional preferred embodiment, amplification is also conducted in the presence of modified nucleotides.

A Subtraction Method for Aptamer Preparation

It is often advantageous in enhancing the specificity of the aptamer obtained to remove members of the starting oligonucleotide mixture which bind to a second substance from which thrombin is to be distinguished. In such subtraction methods, at least two

containing covalently attached thrombin (see, Ellington, A.D., et al., Nature (1990) 346:818-822) or to thrombin in solution (see Blackwell et al., Science (1990) 250:1104-1110; Blackwell et al., Science (1990) 250:1149-1151; or to thrombin bound to a filter (see Tuerk, C., and Gold, L., Science (1990) 249:505-510). Complexes between the aptamer and thrombin are separated from uncomplexed aptamers using any suitable technique, depending on the method used for complexation. For example, if columns are used, non-binding species are simply washed from the column using an appropriate buffer. Specifically bound material can then be eluted.

If binding occurs in solution, the complexes can be separated from the uncomplexed oligonucleotides using, for example, the mobility shift in electrophoresis technique (EMSA), described in Davis, R.L., et al., Cell (1990) 60:733. In this method, aptamer-thrombin complexes are run on a gel and aptamers removed from the region of the gel where thrombin runs. Unbound oligomers migrate outside these regions and are separated away. Finally, if complexes are formed on filters, unbound aptamers are eluted using standard techniques and the desired aptamer recovered from the filters.

In a preferred method, separation of the complexes involves detachment of thrombin-aptamer complexes from column matrices as follows.

A column or other support matrix having covalently or noncovalently coupled thrombin is synthesized. Any standard coupling reagent or procedure may be utilized, depending on the nature of the support. For example, covalent binding may include the formation of disulfide, ether, ester or amide linkages. The length of the linkers used may be varied by conventional means. Noncovalent linkages include antibody-antigen interactions, protein-sugar interactions, as between, for

mercaptoethanol. Thrombin bound to lectin supports may be removed by adding a complementary monosaccharide (e.g., α-methyl-mannoside, N-acetyl glucosamine, glucose, N-acetyl galactosamine, galactose or other saccharides for concanavalin A). Oligonucleotides specifically bound to thrombin can then be recovered by standard denaturation techniques such as phenol extraction.

The method of elution of thrombin-oligonucleotide complex from a support has superior unexpected properties when compared with standard oligonucleotide elution techniques. This invention is not dependent on the mechanism by which these superior properties occur. However, without wishing to be limited by any one mechanism, the following explanation is offered as to how more efficient elution is obtained. Certain support effects result from the binding of oligonucleotides to the support, or the support in conjunction with oligonucleotide or thrombin. Removing oligonucleotide-thrombin complexes enables the recovery of oligonucleotides specific to thrombin only, while eliminating oligonucleotides binding to the support, or the support in conjunction with oligonucleotide or thrombin. At each cycle of selection, this method may give up to 1,000-fold enrichment for specifically binding species. Selection with thrombin remaining bound to support gives less enrichment per cycle, making it necessary to go through many more cycles in order to get a good aptamer population.

Aptamer Pools of Varying Length

Aptamers can also be selected in the above methods using a pool of oligonucleotides that vary in length as the starting material. Thus, several pools of oligonucleotides having random sequences are synthesized that vary in length from e.g. 50 to 60 bases for each

the aptamer is to be used for separation of thrombin, conventionally the oligonucleotide will be derivatized to a solid support to permit chromatographic separation. If the oligonucleotide is to be used for attaching a detectable moiety to thrombin, the oligonucleotide will be derivatized to include a radionuclide, a fluorescent molecule, a chromophore or the like. If the oligonucleotide is to be used in specific binding assays, coupling to solid support or detectable label, and the like are also desirable. If it is to be used in therapy, the oligonucleotide may be derivatized to include ligands which permit easier transit of cellular barriers, toxic moieties which aid in the therapeutic effect, or enzymatic activities which perform desired functions at the thrombin site. The aptamer may also be included in a suitable expression system to provide for in situ generation of the desired sequence.

Consensus Sequences

When a number of individual, distinct aptamer sequences for thrombin have been obtained and sequenced as described above, the sequences may be examined for "consensus sequences." As used herein, "consensus sequence" refers to a nucleotide sequence or region (which may or may not be made up of contiguous nucleotides), which is found in one or more regions of at least two aptamers, the presence of which may be correlated with aptamer-to-thrombin-binding or with aptamer structure.

A consensus sequence may be as short as three nucleotides long. It also may be made up of one or more noncontiguous sequences with nucleotide sequences or polymers of hundreds of bases long interspersed between the consensus sequences. Consensus sequences may be identified by sequence comparisons between individual

one or more of the modified bases, sugars and linkages described herein using conventional techniques and those described herein.

Utility of the Aptamers

The aptamers of the invention are useful in diagnostic, research and therapeutic contexts. For therapeutic applications, the thrombin aptamers have *in vivo* and *ex vivo* clinical utilities, as indicated above. By way of example, the aptamers may be used in the treatment or prevention of (i) restenosis or myointimal thickening associated with angioplasty, (ii) accelerated atherosclerosis after heart transplant operations, (iii) vascular graft reocclusion associated with vascular shunt implants, (iv) clotting or thrombus formation at the site of indwelling arterial or venous access lines, (v) thrombus formation associated with cardiopulmonary bypass surgery, (vi) thrombus formation associated with extracorporeal circuits that are used during various *ex vivo* procedures such as blood dialysis or apheresis, (vii) sepsis-related disseminated intravascular coagulation and (viii) coagulation in patients with known heparin allergy or heparin-induced thrombocytopenia.

For diagnostic applications, these aptamers are well suited for binding to biomolecules that are identical or similar between different species, where standard antibodies may be difficult to obtain. They are also useful in inhibition assays when the aptamers are chosen to inhibit the biological activity of thrombin. Antibodies are generally used to bind analytes that are detected or quantitated in various diagnostic assays. Aptamers represent a class of molecules that may be used in place of antibodies for *in vitro* or *in vivo* diagnostic and purification purposes.

invention are tolerant of harsh chemical conditions, including conditions under which they are synthesized, facile radiolabeling of thrombin aptamers can be conducted without regard to loss of aptamer structure. Only the chemical integrity of the aptamer molecule must be preserved. The aptamers of the invention can be denatured without loss of their capacity to bind thrombin once placed under physiological conditions. Antibodies cannot be reversibly denatured in this manner.

Another consideration relevant to the use of monoclonal antibodies (MAbs) for in vivo imaging is their antigenicity. MAbs are usually derived from mouse hybridomas and as such are foreign proteins. When used in humans they elicit immune responses that limits their use in individual patients to one or two exposures. Once immunized, anti-MAb antibodies generated by an immunized individual leads to rapid clearance of the MAb. This consideration is also relevant to "humanized" MAbs that contain both mouse and human protein sequences.

In addition to chemical stability, the aptamers described herein have a short half-life, a property that can permit rapid in vivo imaging after administration of labeled compound. The thrombin aptamers can also be advantageously used to avoid anaphylactic reactions such as those associated with imaging procedures that use conventional ionic or nonionic contrast agents. The aptamers also have a low molecular weight compared to Abs, which can facilitate their penetration of a target structure, such as a clot, for imaging purposes.

Radiolabeled thrombin aptamers can be used to image arteries or veins according to various clinical indications. For example, such aptamers can be used after angioplasty to image clots, including deep vein clots, CNS thromboses, pulmonary emboli, brain thromboses and the like.

generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition. In general, the dosage required for therapeutic efficacy will range from about 0.1 μg to 20 mg aptamer/kg body weight. Alternatively, dosages within these ranges can be administered by constant infusion over an extended period of time, usually exceeding 24 hours, until the desired therapeutic benefits have been obtained.

For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the aptamers of the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the aptamers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

Systemic administration can also be by transmucosal or transdermal means, or the oligomers can be administered orally. Additional formulations which are suitable for other modes of administration include suppositories, intranasal and other aerosols. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays, for example, or using suppositories. For oral administration, the oligomers are formulated into conventional oral administration forms such as capsules, tablets, and tonics.

Cat. No. NEF-707). The biotin phosphoramidite is incorporated into the strand during solid phase DNA synthesis using standard synthesis conditions.

In another, similar experiment, a pool of 98-mers with the following sequence was synthesized:

5' HO-AGAATACTCAAGCTTGCCG-N_{60}-ACCTGAATTCGCCCTATAG-OH 3'.

DNA 19-mers with the following sequences can also be used as primers for PCR amplification of oligonucleotides recovered from selection columns. The 3' primer sequence is

5' biotin-O-CTATAGGGCGAATTCAGGT-OH 3'

and the 5' primer sequence is

5' HO-AGAATACTCAAGCTTGCCG-OH 3'.

It will be noted that in all cases, the duplex form of the primer binding sites contain restriction enzyme sites.

B. Isolation of Thrombin Aptamers Using Thrombin Immobilized on a Lectin Column

A pool of aptamer DNA 96 bases in length was synthesized as described in Example 1-A, and then PCR-amplified to construct the initial pool. A small amount of the enzymatically-synthesized DNA was further amplified in the presence of α-^{32}P-dNTPs to generate labeled aptamer to permit quantitation from column fractions.

A thrombin column was prepared by washing 1 ml (58 nmole) agarose-bound concanavalin A ("Con-A") (Vector Laboratories, cat. no. AL-1003) with 20 mM Tris-acetate

were low and relatively constant. After recovery of fraction 12, the column was washed with 0.5 mℓ aliquots of 0.1 M α-methyl-mannoside (Sigma Cat. no. M-6882) in selection buffer to elute the bound thrombin along with thrombin-bound aptamers. Fractions 14 and 15 showed a significant peak of thrombin enzyme activity, as determined spectrophotometrically by conversion of a chromogenic substrate (Kabi Diagnostica, Cat. no. S-2238). 0.01% of the input DNA eluted in these two fractions.

Aptamer DNA (Round 1 DNA) was recovered from the thrombin by phenol extraction (2 x 0.5 mℓ). The aqueous phase volume was reduced to about 250 μl by n-butanol extraction. Aptamer DNA was precipitated on dry ice using 3 volumes of ethanol and 20 μg of glycogen as a carrier. The DNA was pelleted, washed once in 70% ethanol and then dried.

C. Amplification of Selected Thrombin Aptamers

Round 1 DNA from Example 1-B was resuspended in 100 μl of H_2O and amplified by PCR. A 200 μl PCR reaction consisted of the following: 100 μl template 96-mer DNA (approximately 0.01 pmoles); 20 μl 10X buffer (100 mM Tris·Cl (pH 8.3), 500 mM KCl, 20 mM $MgCl_2$); 32 μl dNTP's (5 mM conc total, 1.25 mM each dATP, dCTP, dGTP, and dTTP); 20 μl primer 1 (biotinylated 18-mer, 50 μM); 20 μl primer 2 (18-mer, 50 μM); 6μl α-^{32}P-dNTP's (approximately 60 μCi); and 2 μl Taq I Polymerase (10 units). The reaction was covered with 2 drops NUJOL mineral oil. A control reaction was also performed without template aptamer.

Initial denaturation was at 94°C for 3 minutes, but subsequent denaturation after each elongation reaction lasted 1 minute. Primer annealing occurred at 60°C for 1 minute, and elongation of primed DNA strands

analytical PAGE. The resulting amplified Round 1 Pool was applied to a new Con-A-thrombin column as in Example 1-B to obtain Round 2 aptamers.

D. Characterization of Round 1 Through Round 5 Thrombin Aptamers Obtained from Selection on Lectin Columns

Five rounds of thrombin aptamer selection and amplification were carried out using Con-A-thrombin columns as in Examples 1-B and 1-C. As shown in Table 1, the combined fractions 14 and 15 contained a maximum of about 10% of input DNA using the described conditions.

Table 1

Round	% DNA eluted by α-methyl-mannoside*	% DNA bound to control support
1	0.01	0.7
2	0.055	1.9
3	5.80	2.3
4	10.25	1.1
5	9.70	1.0

* 0.1 M α-methyl-mannoside in selection buffer was added as fraction 13 in each elution, and fractions 14 and 15 were retained and the DNA amplified. Due to slow leeching of thrombin from the column, DNA bound to thrombin could also be seen in earlier fractions in rounds 3-5.

After amplification, round 5 aptamer DNA was analyzed for specificity in a filter binding assay. In this assay, nitrocellulose filters (1 cm diameter) prebound with salmon sperm DNA were used to bind either: (1) An unselected 96-mer oligonucleotide DNA pool, (2)

of the resulting clones were then amplified directly using the following 5' primer sequence:

5' HO-CTGCAGGTCGACGCTAGC-OH 3'

and the 3' biotinylated 18-mer primer sequence shown in Example 1-A, and then sequenced.

Filter binding assays using aptamer DNA from 14 of the clones were used to determine the dissociation constants (Kd) for thrombin as follows: Thrombin concentrations between 10 μM and 1 nM were incubated at room temperature in selection buffer for 5 minutes in the presence of 0.08 pmole of radiolabeled 96-mer derived from cloned Round 5 aptamer DNA. After incubation, the thrombin and aptamer mixture was applied to nitrocellulose filters (0.2 micron, 2.4 cm diameter) that were pretreated with salmon sperm DNA (1 mg/mℓ DNA in selection buffer) and washed twice with 1 mℓ selection buffer. After application of thrombin mixture, the filters were washed three times with 1 mℓ selection buffer The radioactivity retained on the filters was then determined. Kd values for the individual clones ranged from 50 to >2000 nM.

The DNA sequence of the 60-nucleotide randomly-generated region from 32 clones was determined in order to examine both the heterogeneity of the selected population and to identify homologous sequences. Sequence analysis showed each of the 32 clones to be distinct. However, striking sequence conservation was found. The hexamer 5' GGTTGG 3' was found at a variable location within the random sequence in 31 of 32 clones, and five of the six nucleotides are strictly conserved in all 32. Additionally, in 28 of the 32 clones a second hexamer 5' GGNTGG 3', where N is usually T and never C,

or a control 7-mer with the same base composition but different sequence (5' GGGGGTT 3'). Clotting times were measured using the timer apparatus as above. The thrombin clotting time in this experiment was 24 sec using thrombin alone (10 nM), 26 sec with thrombin and the control sequence at 20 μM and 38 sec with thrombin plus the consensus sequence at 20 μM, indicating specificity for thrombin inhibition at the level of the 7-mer.

The inhibitory aptamers were active at physiological temperature under physiologic ion conditions and were able to bind to thrombin in the presence of the fibrinogen substrate, a key requirement for therapeutic efficacy.

Example 2

Modified Thrombin Aptamers

Modified forms of the single-stranded, thrombin consensus sequence-containing deoxynucleotide 15-mer described in Example 2, 5' GGTTGGTGTGGTTGG 3', and a closely related 17-mer, were synthesized using conventional techniques. These aptamers for the most part contain the identical nucleotide sequences, bases, sugars and phosphodiester linkages as conventional nucleic acids, but substitute one or more modified linking groups (thioate or MEA), or modified bases (uracil or 5-(1-pentynyl-2'-deoxy)uracil). The aptamers containing 5-(1-pentynyl)-2'-deoxyuridine were generated by replacing thymidine in the parent aptamers. Thrombin aptamers containing 5-(1-pentynyl)-2'-deoxyuridine were also obtained by selection as described in Examples 8 and 9 below.

Independent verification of the Ki for the nonmodified 15-mer was made by determining the extent of thrombin inhibition with varying DNA concentration. The

PCR primer sequences at both the 5′ and 3′ ends and a random 60-mer sequence in the center of the oligomer. Details of synthesis of the pool of single-stranded DNA is disclosed in Example 1 above. PCR conditions were the same as those described above, with the following changes. dATP, dGTP and dCTP were all used at a concentration of 200 μM. The optimal concentration for synthesis of full-length 96-mer DNA via PCR using 5-(1-pentynyl)-2′-deoxyuridine was 800 μM. Generation of PCR-amplified fragments demonstrated that the Taq polymerase both read and incorporated the base as a thymidine analog. Thus, the analog acted as both substrate and template for the polymerase. Amplification was detected by the presence of a 96-mer band on an EtBr-stained polyacrylamide gel.

Example 4

Incorporation of Other Base Analogs Into Candidate Aptamer DNA

Ethyl, propyl and butyl derivatives at the 5-position of uridine, deoxyuridine, and at the N^4-position of cytidine and deoxycytidine are synthesized using methods described above. Each compound is converted to the triphosphate form and tested in the PCR assay described in Example 1 using an appropriate mixture of three normal deoxytriphosphates or ribotriphosphates along with a single modified base analog.

This procedure may also be performed with N^6-position alkylated analogs of adenine and deoxyadenine, and the 7-position alkylated analogs of deazaguanine, deazadeoxyguanine, deazaadenine and deazadeoxyadenine, synthesized using methods described in the specification.

described in Eritja, R., et al, Nucleosides and Nucleotides (1987) 6:803-814. The N,N-diisopropylamino cyanoethylphosphoramidite synthon was prepared by standard methods as described in Caruthers, M.H. Accounts Chem. Res. (1991) 24:278-284, and the derivatized CGP support was prepared by the procedures described in Dahma, M.J., et al, Nucleic Acids Res. (1990) 18:3813. The abasic residue was singly substituted into each of the 15 positions of the 15-mer. Control unmodified aptamer DNA was used as a positive control. The results that were obtained are shown in Table 5.

al, Nucleosides and Nucleotides (1987) 6:287-291; and Froehler, B.C., et al, Tet. Lett. (1986) 27:469. This analog residue was substituted at the indicated positions and the aptamer assayed for inhibition of thrombin. The results that were obtained are shown in Table 6.

Table 6

Compound	clot time (sec) 100 nM	0 nM
GGTTGGTGTGGTZGG	147	-
GGTTGGTGTGGZTGG	129	-
GGTTGGTGZGGTTGG	120	-
GGTTGGZGTGGTTGG	118	-
GGTZGGTGTGGTTGG	187	-
GGZTGGTGTGGTTGG	138	-
GGTTGGTGTGGTTGG	125	-
NO DNA CONTROL	-	23

Z - indicates a 5-propynyl-2'-deoxyuridine residue

Example 8

Incorporation of 5-(1-pentynyl)-2'-deoxyuridine Into Aptamer Candidate DNA

5-(1-pentynyl)-2'-deoxyuridine was synthesized and converted to the triphosphate as described in Otvos, L., et al., Nucleic Acids Res (1987) 1763-1777. The pentynyl compound was obtained by reacting 5-iodo-2'-deoxyuridine with 1-pentyne in the presence of a palladium catalyst. 5-(1-pentynyl)-2'-deoxyuridine triphosphate was then used as a replacement for thymidine triphosphate in the standard PCR reaction.

A pool of 60-mer single-stranded DNA was synthesized, each strand consisting of specific 18-mer PCR primer sequences at both the 5' and 3' ends and a

Thrombin immobilized on a Con-A lectin column served as the target as described.

After five rounds of selection, aptamer DNA was recovered and amplified using thymidine triphosphate (dTTP) in place of 5-(1-pentynyl)-2'-deoxyuridine in order to facilitate subsequent cloning and replication of aptamer DNA in E. coli. At this stage, the presence of a thymidine nucleotide at a given location in an aptamer corresponded to the location of a 5-(1-pentynyl)-2'-deoxyuridine nucleotide in each original round five aptamer. Thus, dTTP served to mark the location of 5-(1-pentynyl)-2'-deoxyuridine residues in the original selected DNA pools.

The round five amplified DNA containing dTTP was digested with BamHI and HindIII and cloned into the corresponding sites of pGEM 3Z (Promega Biotech) and transformed into E. coli. DNA from 21 clones was analyzed by dideoxy sequencing. Three of the clones contained aptamer sequences that were identical. Only one of the 21 clones contained a sequence that closely resembled the original 5' GGTTGG 3' binding motif obtained using thymine in the selection protocol.

One of these two clones (#17) and the original unselected pool was analyzed for thrombin binding by nitrocellulose filter assay described above using DNA labeled with ^{32}P to permit analysis of thrombin binding characteristics. The labeled DNA was synthesized by PCR and contained 5-(1-pentynyl)-2'-deoxyuridine in order to retain the original selected DNA structures. The DNA was incubated with thrombin at various concentrations between 10 nM and 10 μM to obtain the Kd values for thrombin binding. The Kd of the unselected pool was >10 μM while the Kd of clone 17 was 300 nM.

Radiolabeled clone 17 DNA was synthesized using thymidine in place of 5-(1-pentynyl)-2'-deoxyuridine and

mM NaCl and 200 μM of each of dATP, dGTP, dTTP and 5-methyl-2'-deoxycytidine triphosphate. 20 μCi each of α-^{32}P-dATP and dGTP were added to label the DNA. 1 nmole of 5' and 3' primer were added followed by addition of 0.2 pmole of 98-mer template pool DNA. Amplification was initiated by addition of 2 μL (10 U) of Taq polymerase followed by sealing of the reaction with a mineral oil overlay. About 16 cycles of amplification were performed followed by a 10 minute final extension to complete all duplex synthesis.

Amplified DNA was recovered (100 μL aqueous phase), n-butanol extracted (650 μL) and applied to a Nick column prewashed with 5 mL of buffer containing 100 mM Tris-HCl pH 7.5 and 100 mM NaCl. Eluted DNA was applied to a 0.5 mL avidin-agarose column prewashed in the same buffer and washed until DNA loss from the column was < 1000 cpm. Single stranded DNA was eluted from the avidin column by washing with 0.15 N NaCl and the eluate was neutralized to pH 7.0 using glacial acetic acid. The 98-mer DNA was exchanged into selection buffer on a second Nick column and, after heat denaturation for 3 min at 95° C followed by cooling on ice for 10 min, used in aptamer selection on thrombin lectin columns. 1 mL thrombin columns were equilibrated in selection buffer prior to addition of single-stranded DNA. The single-stranded DNA was recirculated for three complete passes. Upon completion of the third pass the peak radioactive element was then applied to a 1 mL ConA/thrombin column (charged with 3 nmoles of thrombin). Radioactive single-stranded 98-mer was applied three times to this matrix. At the third application, the column was stoppered and allowed to stand for 1 hr. The column was then washed with selection buffer and 0.5 mL aliquot fractions collected. A total wash volume of 6 mL was employed. At this time, 0.1 M α-methyl-mannoside in selection buffer

radiolabeled aptamer DNA at about a concentration of about 1 nM was incubated with the indicated protein for several minutes at room temperature, followed by filtration of the aptamer-protein mixture through a nitrocellulose filter. The filter was washed with 3 mL of selection buffer and then radioactivity bound to the filters was determined as a % of input radioactivity. Results obtained are shown in Table 7. Binding data is shown for both unselected 96-mer DNA and for two separate experiments with clone #29 96-mer. All proteins were tested at about 1μM concentration except human serum albumin which was used at 100 μM. The results that were obtained demonstrated that the 96-mer specifically bound to thrombin and had little affinity for most of the other proteins tested.

The thrombin 21-mer ancrod assay was conducted as follows. Ancrod was suspended in sterile water at a concentration of 44 U/mL. 10 μL ancrod solution was added to 95 μL of selection buffer prewarmed to 37°C. 100 μL of this mixture was transferred to the coagulation cup of the fibrometer described above, followed by addition of 200 μL of fibrinogen and 20 μL of 21-mer DNA (both prewarmed to 37°C). TE buffer pH 7.0 was used as a control lacking DNA. The control clot time was 25 seconds while the clot time in the presence of 500 nM 21-mer was 24 seconds and was 26 seconds in the presence of 33 μM 21-mer. This result demonstrated the specificity on inhibition of fibrinogen cleavage was limited to thrombin; ancrod was not affected.

Example 12

Thrombin Aptamer Pharmacokinetic Studies

A 15-mer single-stranded deoxynucleotide, 5' GGTTGGTGTGGTTGG 3', identified as a consensus sequence from 30 thrombin aptamer clones as described in Example 1 above, was used. Young adult rats of mixed gender and strain were used. The animals were anaesthetized and a diester of the 15-mer was injected through a catheter in 200 μl volumes (in 20 mM phosphate buffer, pH 7.4, 0.15 M NaCl) at two concentrations, so that the final concentration of 15-mer in the blood was about 0.5 and 5.0 μM respectively, although the exact concentration depends on the volume of distribution (which is unknown for this oligonucleotide). These values are 10 to 100 times greater than the human in vitro Kd value. No heparin was used for catheterization.

At 0, 5, 20 and 60 minutes, blood was withdrawn from the animals (approx. 500 μl aliquots), transferred into tubes containing 0.1 volume citrate buffer, and centrifuged. Rat plasma was removed and tested in a

using the plasma in the PT test. Control thrombin PT clot time values were obtained several minutes prior to administration of aptamer. Briefly, the PT assay was conducted using 0.1 mL of monkey plasma prewarmed to 37° C and 0.2 mL of a 1:1 mixture of thromboplastin (used according to manufacturers instructions) and $CaCl_2$ (25 mM), also prewarmed to 37°C. Thrombin clot times were measured with a fibrometer as described above.

The animals were at least two years old and varied in weight from 4 to 6 kg. Doses of aptamer were adjusted for body weight. Aptamer DNA was dissolved in sterile 20 mM phosphate buffer (pH 7.4) at a concentration of 31.8 to 33.2 mg/mL and diluted in sterile physiological saline prior to delivery. Bolus injections were administered to give a final concentration of 22.5 mg/Kg (1 animal) of the diester aptamer or 11.25 mg/Kg (1 animal) of the diester aptamer. Infusions were administered over a 1 hour period to three groups of animals: (i) 0.5 mg/kg/min of diester 15-mer (4 animals), (ii) 0.1 mg/kg/min of diester 15-mer (2 animals) and (iii) 0.5 mg/kg/min of thioate analog 15-mer (2 animals).

PT assay results from the bolus injections showed thrombin inhibition times of 7.8, 3.3 and 1.35 times control at 2.5, 5.0 and 10.0 min respectively after delivery of the aptamer for the high dose animal. Inhibition times of 5.6, 2.2 and 1.2 times control were obtained from the low dose animal at the same time points.

Figure 2 shows a plot of the PT times from the 4 animals that received the high dose diester infusion compared to pretreatment control values. The data points show the PT clot time as an average value obtained from the 4 animals in the group. The arrows indicate time points at the beginning and end of the infusion period.

until the experiment was terminated at 80 minutes after start of circulation in the unit. Blood coagulation occurred at 51 minutes in one trial with the 15-mer. In a second trial, coagulation did not occur during the 80 minute course of the experiment.

Thus, methods for obtaining aptamers that specifically bind thrombin are described, as well as the therapeutic utility of these aptamers and the use of the aptamers in the detection and isolation of thrombin. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and scope of the appended claims.

9. The aptamer of claim 8 wherein said binding region contains more than 5 and less than 16 nucleotide residues.

10. The aptamer of claim 1 wherein the aptamer contains at least one modified base, sugar, or linking group.

11. The aptamer of claim 10 wherein

the aptamer contains at least one linking group wherein P(O)O is replaced by P(O)S, P(S)S, P(O)NR_2, P(O)R, P(O)OR', CO or CH_2, wherein each R or R' is independently H or substituted or unsubstituted alkyl (1-20C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or aralkyl; or

the aptamer contains at least one linking group attached to an adjacent nucleotide through S or N; or

the aptamer contains at least one analogous form of purine or pyrimidine, or at least one abasic site.

12. The aptamer of claim 11 which is a single-stranded DNA.

13. The aptamer of claim 11 which contains at least one linking group wherein P(O)O is replaced by P(O)S, and wherein said linking group is attached to each adjacent nucleotide through O.

14. The aptamer of claim 11 which contains at least one linking group wherein P(O)O is replaced by P(O)NH($CH_2CH_2OCH_3$), and wherein said linking group is attached to each adjacent nucleotide through O.

24. The aptamer of claims 1-23 wherein said binding region comprises the sequence GGXTGG, wherein X is T, A, U, dU or G.

25. The aptamer of claim 24 wherein said nucleotide sequence has the formula GGTTGG.

26. The aptamer of claim 24 wherein said thrombin binding region comprises the sequence GGXTGG$(N)_z$GGXTGG or a fragment thereof, wherein N is G, A, C, U, dU or T, and z is an integer from 2 to 5.

27. The aptamer of claim 26 wherein said sequence has the formula GGTTGGTGTGGTTGG.

28. The aptamer of claim 27 having the formula: GGTTGGTGTGGTTGG#G#T wherein # denotes an MEA linkage.

29. The aptamer of claim 27 having the formula GGTTGGTGTGGTT*G*G wherein * denotes a thioate linkage.

30. The aptamer of claim 27 having the formula G*G*T*T*G*G*T*G*T*G*G*T*T*G*G wherein * denotes a thioate linkage.

31. The aptamer of claim 27 having the formula GGTTGG(dU)G(dU)GGTTGG.

32. The aptamer of claim 27 having the formula GG(dU)TGGTGTGG(dU)TGG.

33. The aptamer of claim 27 having the formula GGTTGGTGTGGTU′GG wherein U′ denotes 5-pentynyluracil.

44. The aptamer of claims 1-43 wherein the Kd with respect to the aptamer and thrombin is less by a factor of at least 5, as compared to the Kd for said aptamer and other molecules.

45. The aptamer of claims 1-44 which is a secondary aptamer.

46. A method for obtaining an aptamer containing at least one binding region that specifically binds thrombin, which method comprises:

(a) incubating thrombin with a mixture of oligonucleotides under conditions wherein complexation occurs with some, but not all, members of the mixture to form oligonucleotide-thrombin complexes;

(b) separating the oligonucleotide-thrombin complexes from uncomplexed oligonucleotide;

(c) recovering and amplifying the complexed oligonucleotide from said complexes; and

(d) optionally determining the sequence of the recovered oligonucleotide.

47. The method of claim 46 wherein said aptamer is a single-stranded DNA, or

wherein said aptamer contains at least one binding region capable of binding specifically to thrombin with a dissociation constant (Kd) of less than 30×10^{-9}, or

wherein said aptamer contains at least one binding region capable of binding specifically to thrombin wherein the Kd with respect to the aptamer and thrombin is less by a factor of at least 10, as compared to the Kd for said aptamer and other molecules, or

complexation occurs with some, but not all, members of the mixture to form oligonucleotide-thrombin complexes;

(b) separating the oligonucleotide-thrombin complexes from uncomplexed oligonucleotides;

(c) recovering and amplifying the complexed oligonucleotides from said complexes;

(d) optionally repeating steps (a)-(c) with the recovered oligonucleotides of step (c);

(e) determining the sequences of the recovered oligonucleotides;

(f) determining a consensus sequence included in the recovered oligonucleotides; and

(g) synthesizing a secondary aptamer which comprises the consensus sequence.

55. A secondary aptamer prepared by the method of claim 54.

56. A complex formed by thrombin and the aptamer of claims 1-45, 53, or 55.

57. A method for obtaining an aptamer containing at least one binding region that specifically binds thrombin, which method comprises:

(a) incubating thrombin with a mixture of oligonucleotides under conditions wherein complexation occurs with some, but not all, members of the mixture to form oligonucleotide-thrombin complexes;

(b) separating the oligonucleotide-thrombin complexes from uncomplexed oligonucleotide;

(c) recovering and amplifying the complexed oligonucleotide from said complexes; and

(d) optionally determining the sequence of the recovered oligonucleotide,

amplified complexed oligonucleotides resulting from step (c) in succeeding step (a).

64. An aptamer prepared by the method of claims 57-63.

65. A method to detect the presence or absence of thrombin, which method comprises contacting a sample suspected of containing thrombin with the aptamer of claims 1-45 under conditions wherein a complex between thrombin and the aptamer is formed, and

detecting the presence or absence of said complex.

66. A method to purify thrombin, which method comprises contacting a sample containing thrombin with the aptamer of claims 1-45 attached to solid support under conditions wherein thrombin is bound to the aptamer coupled to solid support;

washing unbound components of the sample; and

recovering thrombin from said solid support.

67. A pharmaceutical composition for medical use comprising the aptamer of claims 1-45 in admixture with a physiologically acceptable excipient.

68. A composition for diagnostic use which comprises the aptamer of claims 1-45.

69. The aptamer of claims 1-45 coupled to an auxiliary substance.

70. The aptamer of claim 69 wherein said auxiliary substance is selected from the group consisting

75. The method of claim 74 wherein the monosaccharide is selected from the group consisting of α-methyl-mannoside, N-acetylglucosamine, glucose, N-acetylgalactosamine and galactose.

76. The method of claim 75 wherein the support is a concanavalin A column.

77. A composition for use in binding or inhibiting thrombin which composition comprises an aptamer as described in claims 1-45.

77. A composition for use in inhibiting clotting or coagulation in a patient's blood which composition comprises an aptamer as described in claims 1-45.

78. A composition for use in inhibiting or reducing restenosis, which composition comprises an aptamer as described in claims 1-45.

79. A composition for use in treating a patient's blood <u>ex corpore</u> to inhibit clot formation, which composition comprises an aptamer as described in claims 1-45.

80. A method to prevent coagulation during cardiopulmonary bypass surgery, which method comprises contacting blood with an aptamer as described in claims 1-45.

81. In a method to inhibit clot formation which comprises contacting blood with a fibrinolytic agent, the improvement which comprises:

1/2

#		•																			•	
1		26		G	G	G	T	T	G	G	-	-	G	T	C	G	G	T	T	G	G	T
2		20		G	G	G	A	T	G	G	-	-	T	T	T	G	G	T	T	G	G	G
3		32		A	G	G	T	T	G	G	-	-	-	G	A	G	G	G	T	G	G	G
4		41		T	G	G	T	T	G	G	-	-	C	G	A	G	G	A	T	G	G	A
5		56		A	G	G	T	T	G	G	-	G	T	A	G	T	G	T	T	G	G	T
6		29		A	G	G	T	T	G	G	-	-	G	C	T	G	G	T	T	G	G	G
7		17		G	G	G	T	T	G	G	-	-	-	G	A	G	G	T	T	G	G	A
8		44		T	G	G	T	T	G	G	-	-	G	T	C	G	G	T	T	G	G	G
9		50		G	G	G	A	T	G	G	-	-	T	G	T	G	G	T	T	G	G	C
10		30		T	G	G	T	T	G	G	-	-	C	A	G	G	G	A	T	G	G	G
11		25		T	G	G	A	T	G	G	-	-	T	G	A	G	G	T	T	G	G	A
12		28		G	G	G	G	T	G	G	-	-	T	T	A	G	G	T	T	G	G	T
13		47		A	G	G	G	T	G	G	-	-	T	T	A	G	G	T	T	G	G	T
14		42		C	G	G	T	T	G	G	-	G	T	T	G	G	G	A	T	G	G	A
15		41		C	G	G	T	T	G	G	-	-	T	G	T	G	G	T	T	G	G	T
16		53		A	G	G	T	T	G	G	-	-	T	G	T	G	G	G	T	G	G	G
17		15		C	G	G	G	T	G	G	-	-	A	T	A	G	G	T	T	G	G	A
18		24		G	G	T	G	T	G	G	T	A	G	T	T	T	G	T	T	G	G	G
19		23		T	G	G	T	T	G	G	T	T	A	C	T	G	G	T	T	G	G	G
20		27		G	G	G	T	T	G	G	-	-	T	C	T	G	G	G	T	G	G	A
21		36		T	G	G	T	T	G	G	-	-	G	T	T	G	G	G	T	G	G	A
22		25		T	G	G	T	T	G	G	-	-	C	C	A	G	G	T	T	G	G	A
23		12		C	T	A	G	C	G	G	-	C	A	G	T	G	G	T	T	G	G	G
24		25		T	G	G	G	T	G	G	-	-	G	G	A	G	G	T	T	G	G	T
25		49		A	G	G	T	T	G	G	-	-	T	T	T	G	G	G	T	G	G	T
26		28		A	G	G	T	T	G	G	-	T	T	A	G	G	G	T	T	G	G	T
27		18		G	G	G	A	T	G	C	-	-	G	G	T	G	G	T	T	G	G	G
28		55		T	G	G	T	T	G	G	-	T	T	A	T	G	G	T	T	G	G	T
29		23		A	G	G	T	T	G	G	-	-	T	G	T	G	G	T	T	G	G	C
30		40		A	G	G	T	T	G	G	-	-	T	G	T	G	G	G	T	G	G	G
31		41		T	G	G	T	T	G	G	-	-	-	G	A	G	G	T	T	G	G	T
32		42		G	G	G	T	T	G	G	T	G	G	G	T	G	G	A	T	G	G	T
											Consensus Sequence											
					G	G	T	T	G	G		(N)3				G	G	T	T	G	G	
	G			9	31	30	6	0	32	31						30	32	6	0	32	32	11
	A			9	0	1	4	0	0	0						0	0	4	0	0	0	8
	T			10	1	1	22	31	0	0						2	0	22	32	0	0	11
	C			4	0	0	0	1	0	1						0	0	0	0	0	0	2

FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01367

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12Q 1/68; C07H 15/12, 17/00
US CL : 435/6, 536/27, 28, 29

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/6, 536/27, 28, 29

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4,647,529 (Rodland et al) 03 March 1987, see abstract and column 10, lines 34-48.	1-76
Y	Nature, Volume 346, Issued 30 August 1990, Ellington et al. "In vitro Selection of RNA Molecules that Bind Specific Ligands," pages 818-822, see entire document.	1-28, 31-63, and 72-76
Y	Proceedings of the National Academy of Sciences, Vol. 82, issued November 1985, Huynh-Dinh et al. "Modified Oligonucleotides as Alternatives to the Synthesis of Mixed Probes for the Screening of cDNA Libraries," pages 7510-7514, see abstract and page 7510, column 1, paragraph 3.	1-76
Y	Science, Volume 249, issued 03 August 1990, Tuerk et al., "Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase," pages 505-510. See entire document.	1-28, 31-64 and 72-76

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
24 APRIL 1992	18 MAY 1992
International Searching Authority[1]	**Signature of Authorized Officer**[20]
ISA/US	Mindy B. Fleisher

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y	Nucleic Acids Research, Volume 18, number 11, issued 1990, Thiesen et al. "Target Detection Assay (TDA): A Versatile Procedure to Determine DNA Binding Sites as Demonstrated on SP1 Protein," pages 3203-3208, see entire document.	1-28, 31-64 and 72-76
Y	Kirk-Othmer, "Encyclopedia of Chemical Technology, Third Edition, Volume 6" published 1979 by John-Wiley and Sons (NY), pages 35-54. See pages 35-54.	72-76
Y	US, A, 4,748,156 (Aoki et al) 31 May 1988, see abstract.	1-28, 31-63, and 72-76